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Evolution of Developmental Control Mechanisms

Short neuropeptide *F* (sNPF) is a stage-specific suppressor for juvenile hormone biosynthesis by corpora allata, and a critical factor for the initiation of insect metamorphosis

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ABSTRACT

Molting and metamorphosis are essential events for arthropod development, and juvenile hormone (JH) and its precursors play critical roles for these events. We examined the regulation of JH biosynthesis by the corpora allata (CA) in *Bombyx mori*, and found that intact brain-corpora cardiaca (CC)–CA complexes produced a smaller amount of JH than that in CC–CA complexes and CA alone throughout the 4th and 5th (last) instar stadium. The smaller amount of synthesis was due to allatostatin-C (AST-C) produced by the brain. The CC synthesized short neuropeptide F (sNPF) that also suppressed the JH synthesis, but only in day 3 4th stadium and after the last larval ecdysis. For the suppression, both peptides prevented the expression of some of the distinct JH biosynthetic enzymes in the mevalonate pathway. Allatotropin (AT) stimulated sNPF expression in the CC of day 1 5th instar stadium, not of day 3 4th; therefore the stage-specific inhibition of JH synthesis by sNPF was partly due to the stimulative action of AT on the sNPF expression besides the stage-specific expression of the sNPF receptors in the CA, the level of which was high in day 2 4th and day 0 5th instar larvae. The cessation of JH biosynthesis in the last instar larvae is a key event to initiate pupal metamorphosis, and both sNPF and AST-C are key factors in shutting down JH synthesis, along with the decline of ecdysone titer and dopamine.

Introduction

Insect molting and metamorphosis are precisely controlled by circulating ecdysone and juvenile hormone (JH) in the hemolymph, i.e. ecdysone induces molting, and JH modulates the ecdysone action, such that JH allows larval molting in response to ecdysone but prevents metamorphosis. Ecdysone synthesis is under the control of prothoracicotropic hormone (PTTH) released from the brain, with minor modifications by peptides such as Bommo-FMRFamide, Bommo-myosuppressin, prothoracicostatic peptide, and orcokinin (reviewed by Yamanaka et al., 2013), and the mechanisms of the most important timings of PTTH release are relatively well documented. JH biosynthesis by the corpora allata (CA) is regulated by both neuronal connections to the brain and several humoral factors (Stay and Tobe, 2007; Goodman and Cusson, 2012), but the regulatory mechanisms of the JH synthesis have been only partially uncovered (Hiruma and Kaneko, 2013).

The disappearance of JH from the hemolymph is essential to initiate insect metamorphosis (Riddiford, 1996; Hiruma, 2003), and the cessation of JH synthesis is initiated in the last instar stadium by surpassing the threshold of a critical weight ensuring the decline of the JH titer by the action of JH esterase (Nijhout and Williams, 1974; Browder et al., 2001). The concentration of JH in the hemolymph is regulated not only by its synthesis but by the degradation, sequestration, secretion, etc. (Goodman and Cusson, 2012) and the most important is the synthesis (Niimi and Sakurai, 1997; Kinjoh et al., 2007). A number of factors are involved in the regulation of JH biosynthesis; such as catecholamines (dopamine and glutamate), cAMP, ecdysone, and peptides including allatostatins (ASTs), allatotropins (ATs), allatoinhibin, etc. (reviewed by Stay and Tobe, 2007, Goodman and Cusson, 2012, Hiruma and Kaneko, 2013). Recent studies in *Bombyx mori* have shown that both ecdysone and dopamine were important factors to regulate JH synthesis in a stage-specific fashion in the 4th and 5th instar stadium (Kaneko et al., 2011b: Hiruma and Kaneko, 2013). On the other hand, among the allatoregulatory peptides ASTs and ATs have been studied well in terms of structure-activity relationships in adult stage; these studies were primarily the isolation, characterization and distribution of these peptides and their gene

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structures in different insect species (Stay and Tobe, 2007; Weaver and Audsley, 2009).

The JH titer in the hemolymph changes during insect development, and understanding the control mechanisms of JH biosynthesis will shed light on the regulation of the changing JH titer that governs insect molting and metamorphosis. Although allatoregulatory peptides would have an important contribution on the JH biosynthesis in each developmental stage, the roles of these peptides in association with the fluctuation of JH titer are yet to be established.

Within Lepidoptera, the brain is known to regulate JH synthesis in both *Manduca sexta* and *B. mori* (Whisenton et al., 1985, 1987; Kaneko et al., 2011b), but studies on the roles of the corpora cardiaca (CC) have not been under consideration. The CC are attached to both the brain and the CA by nerves and locate between them, so that they likely influence the JH synthesis. Short Neuropeptide *F* (sNPF) is a small neuropeptide [xPxLRLRFamide] produced by the CC and transferred to the CA in *B. mori*, and its receptor is expressed in the CA where JH is synthesized (Yamanaka et al., 2008). In this paper, we show that not only the brain but the CC are key organs regulating JH biosynthesis *in B. mori,* and sNPF synthesized by the CC is one of the key factors in generating the stage-specific suppression of JH synthesis during larval development. In addition, sNPF is a critical factor to shut-down the JH synthesis in the 5th instar stadium to ensure the initiation of pupal metamorphosis.

Materials and methods

Animals

Larvae of the silkworm, *B. mori* (Kinshu x Showa, F1 hybrid), were reared on an artificial diet (Nihon Nosan Kogyo, Japan) at 25 °C under 12 h light:12 h dark cycle. Under these conditions, 3rd instar larvae ecdysed to 4th (penultimate) instars at the beginning of a scotophase, therefore day 0 of the 4th stadium was only 12 h. Fourth instar larvae ecdysed to 5th instars at the beginning of a photophase; therefore, the first photophase was designated as day 0 5th.

Hormones and in vitro culture

Twenty-hydroxyecdysone (20E) was a gift from Dr. Takeshi Matsumoto (Daicel Chemical Co). It was dissolved into ethanol, and stored at −20 °C. The concentration was determined spectrophotometrically (ε_{240}=12,677 in EtOH). AST-C, sNPFs and AT were a gift from Dr. Naoki Yamanaka and Dr. Hiroshi Kataoka (Tokyo University). These were dissolved into water, and stored at −20 °C.

Two brain–CC–CA complexes, two pairs of CC–CA complexes or two pairs of CA were cultured for 6 h in 50 μl of medium-199 (with Hank's salts, 25 mM HEPES buffer (pH 7.2) and 2% Ficoll 400) with or without peptide hormones. In co-culture experiments, two brains were dissected from the same larvae from which CC–CA or CA were dissected. Two brains were cultured with four CC–CA or four CA in a culture well. All the 96 well culture plates were coated with polyethylene glycol 20,000.

Radiochemical assay for JH biosynthesis

JH biosynthesis was measured by as described (Kinjoh et al., 2007; Kaneko et al., 2011b). Briefly, glands were pre-incubated in Medium-199 without methionine (with Hank's salts, 25 mM HEPES buffer (pH 7.2) and 2% Ficoll 400) for 1 h. They were then transferred into a fresh medium supplemented with 10 μM of the JH esterase inhibitor 3-octylthio-1,1,1-trifluoro-2-propanone (OTFP) (a gift of Dr. Takahiro Shiotsuki) and 74 kBq of L-[methyl-3H]-methionine (2.96 TBq/mmol, final concentration in the medium was 0.48 μM), and incubated for 6 h. After the incubation, the labeled JH was extracted by isooctane and subjected to thin layer chromatography (Merck silica gel 60). The region of JH I, II and III (identified from UV absorbance of authentic internal standards obtained from SciTech, Praha, Czech Republic) was cut out and assayed for radioactivity by a liquid scintillation counter (Lsc-5100, Aloka).

Quantitative real-time PCR

Total RNA was extracted from 60 CC or 30 CA by RNAqueous® Kit (Ambion). After treatment with RNase-free DNase I, complementary DNA (cDNA) was prepared from the total RNA and reverse transcriptase (Invitrogen). Each gene transcript was quantified on a real-time thermal cycler (Lightcycler, Roche). Serial dilutions of plasmid containing the cDNA in which we were interested were used as standards. 1X SYBR Green Premixed Ex Taq (TaKaRa) was used for quantitative real-time PCR, and PCR conditions were 95 °C for 5 s and 60 °C for 20 s for 40 cycles, after one cycle of 95 °C for 10 s. The molar amounts of transcripts were calculated with standard curves generated from the plasmid containing cDNAs. The transcript levels were normalized to those of *rp49*. The primers for acetoacetyl-CoA thiolase (AACT), HMG Co-A synthase (HMGS), HMG Co-A reductase (HMGR), mevalonate kinase (MevK), phosphomevalonate kinase (MevPK), diphosphomevalonate decarboxylase (MevPPD), IPP isomerase (IPPI), farnesyl diphosphate synthase 1 (FPPS1), farnesyl diphosphate synthase 2 (FPPS2), farnesyl diphosphate synthase 3 (FPPS3), and JH acid *O*-methyltransferase (JHAMT) and rp49 were the same as used in Ref. Kinjoh et al. (2007). The primers for AST-C receptor, sNPF, sNPF receptors and AT receptor were as described in Ref. Yamanaka et al. (2008).

Results

Roles of brain and CC on the JH biosynthesis by CA

CC are considered as a neurohemal organ for neurosecretions from the brain. Since the CC are located between the brain and the CA (Kaneko et al., 2011a), it is possible that the CC also have a function regulating JH biosynthesis. To study the roles of the brains and CC on the JH synthesis by the CA, we cultured intact brain–CC–CA complexes, CC–CA complexes without brains, and CA alone (Fig. 1A). The synthetic activity of JH by the brain–CC–CA complex was high during the 4th stadium, and then declined after the last larval ecdysis, followed by its cessation completely by day 2 of the 5th stadium. The synthetic activity of the CA without the brain, either as the CC–CA complexes or the CA alone, was about two-fold higher than that with the brain, and the patterns of the synthetic activity of the CA alone were similar to those of the brain–CC–CA complexes. By contrast, the synthetic activity by the CC–CA complex showed two declines, the first on day 3 4th instar stadium, and the second after ecdysis to the last larval stadium. These results indicate that the brain has an inhibitory action on JH synthesis, and the CC of day 3 4th and the early 5th instar larvae seem to release an inhibitory factor for JH synthesis whose synthesis/release seems to be under the control of the brain.

To study the nature of the inhibition by brains, we co-cultured isolated brains of day 2 4th instar larvae with CA alone or CC–CA complexes to examine whether or not JH synthesis is suppressed. Fig. 1B shows that JH biosynthetic activity was only slightly inhibited in the presence of intact brains, indicating that the inhibition by brains was either due to the nervous inhibition or the release of only a small amount of inhibitory factor(s) into the

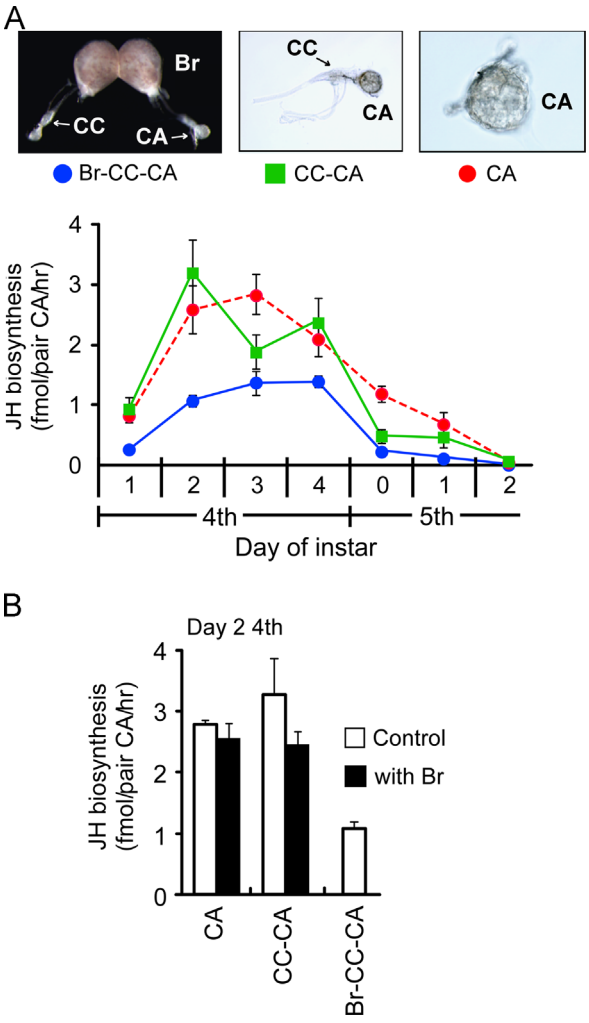

Fig. 1. (A) JH biosynthesis by the Br–CC–CA, CC–CA complexes, and CA alone in vitro during the 4th and early 5th instar stadium. Each point represents Mean ± S.E. (N=3–5). Br–CC–CA, brain–corpora cardiaca–corpora allata; CC–CA, corpora cardiaca–corpora allata; CA, corpora allata. (B) Effect of brains on the JH biosynthesis by CA alone and CC–CA complexes. Two pairs of CA and CC–CA complexes from day 2 4th instar larvae were co-cultured with two brains from the same larvae for 6 h, then JH biosynthesis was determined. The Brain–CC–CA complexes were also cultured for 6 hr for comparison. Each point represents Mean ± S.E. (N=3–15).

culture medium from the brain that was below the threshold level of the inhibitory action. In the latter case, the normal delivery of an inhibitory factor from the brain is likely through the axons to the CA, such that a lower amount is effective.

Inhibition of JH synthesis by allatostatin-C (AST-C) and sNPF

AST-C

When CA from various stages during 4th and 5th instar larvae were cultured with 1 μM AST-C for 6 h, AST-C suppressed the JH biosynthetic activity by 52–82% in all the stages studied (Fig. 2A), and > 0.5 μM of AST-C effectively suppressed its synthetic activity in day 2 4th CA (Fig. 2B). The suppressive action of AST-C was not influenced by the addition of AT in day 0 5th CA (Fig. 5C). Therefore, one of the humoral factors from the brain suppressing JH synthesis is AST-C.

The mRNA of the AST-C receptor in the CA was already present in the early 4th instar stadium and expression increased up to the molting period and to one day after the ecdysis with a small depression shortly before the ecdysis. In the 5th instar, the expression of this mRNA has declined to negligible levels by day 5 (Fig. 2C) and corresponds with the cessation of the JH synthesis by CA (Kinjoh et al., 2007).

sNPF

The synthetic activity of JH by the CA attached to the CC decreased in two different stages, in day 3 4th and at and after the final larval ecdysis (Fig. 1A), and a strong candidate for the inhibitory factor from the CC is sNPF, as it is synthesized by the CC and then transferred to the CA through the nerves (Yamanaka et al., 2008). To determine whether or not sNPF inhibits JH biosynthesis in a stage-specific fashion, CA from various different stages were cultured with 3 μM sNPF (mixture of 1 μM each sNPF-1, -2, and -3; unless otherwise mentioned) for 6 h, and then the synthetic activity of JH was determined. Fig. 3A shows that sNPF significantly suppressed JH synthetic activity in the CA of day 3 4th and of the early 5th instar stadium, which corresponds with the stages the time at which the depression of the JH biosynthesis by the CC–CA (Fig. 1A). In particular, the synthetic activity of JH was strongly suppressed by 82% in day 1 5th CA, and the addition of AT was little affected on its synthetic activity (Fig. 5B). Dose responses showed that greater than 0.75 μM sNPF (as mixed peptide) was effective (Fig. 3B). Co-culture of CC had little effect on the JH synthesis (data not shown, N=10).

Of the three isoforms of sNPF, (sNPF-1, -2, and -3) (Yamanaka et al., 2008), only sNPF-1 and sNPF-2 showed inhibitory activity when assayed using CA of day 0 5th instar larvae, both giving about 50% inhibition of JH biosynthesis at 1 μM (Fig. 3C).

Two isoforms of putative sNPF receptor genes, *BNGR-A10* and *BNGR-A11*, have been isolated and both are expressed in the CA (Yamanaka et al., 2008). When the mRNA levels of each of these receptors in the CA was examined during the 4th and 5th instar larvae, the expression patterns differed; *BNGR-A10* gene expression peaked on day 2–3 4th, then declined and peaked again at the time of and shortly after the last larval ecdysis followed by a sharp decline (Fig. 3D). Yet the expression of the *BNGR-A11* gene began to increase when *BNGR-A10* expression increased again at the time of ecdysteroid titer declined and peaked shortly after the last larval ecdysis, then declined gradually. In addition, the number of the transcripts at the peak levels were three-fold higher in the *BNGR-A10* gene.

The *BNGR-A10* expression declined in day 3–4 of the 4th instar stadium when the ecdysteroid titer increased for larval molting (Kaneko et al., 2011b); therefore one of the factors to cause its decline may be the ecdysteroid titer. Culture of the CA of day 2 4th instar larvae with 0.5 μg/ml 20E for 6 h had little effect on expression of either receptor (Fig. 3E). Yet in the CA of day 0 5th instar, the same treatments caused significant decline of both *BNGR-A10* and *BNGR-A11* transcript levels (Fig. 3E), indicating that they are partly regulated by ecdysone in a stage-dependent manner and the decline of ecdysteroid titer toward the last larval ecdysis (Kaneko et al., 2011b) allows the increase of the transcription of these factors (Fig. 3D).

Mode of suppressive action of AST-C and sNPF on JH biosynthesis

One of the likely mechanisms of the suppressive action of AST-C and sNPF on JH biosynthesis by the CA is the suppression of the JH synthetic enzymes. To verify this hypothesis, CA from the day 0 5th instar larvae were cultured with or without 3 μM sNPF or 1 μM AST-C for 6 h, and then the amounts of transcripts of these enzymes in the CA were measured (Fig. 4). When sNPF was present, the expression levels of HMGR and MevK were significantly suppressed, by 20% and 50%, respectively. On the other hand, AST-C suppressed these same enzymes (HMGR and MevK) together with IPPI, by 50–70% (Fig. 4). These results demonstrate

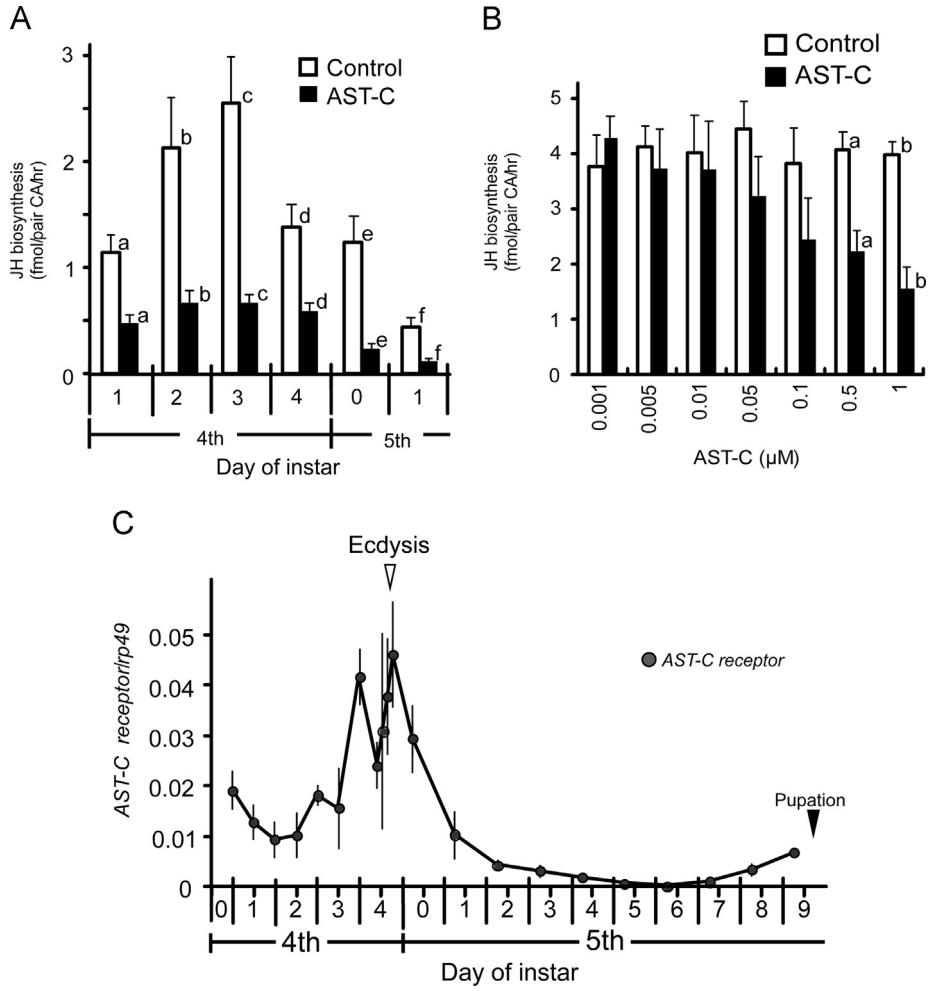

Fig. 2. Inhibition of JH synthesis by AST-C. (A) Developmental changes in allatostatic effects of AST-C on JH biosynthesis. Two pairs of CA were dissected from each day from day 1 4th to day 1 5th were cultured with or without 1 μM AST-C for 6 h, then the rate of JH biosynthesis was determined. Each point shows Mean ± S.E. (N=4–6). [a, b, c, d, e, f] Significantly different at P=0.0074, 0.0254, 0.0016, 0.022, 0.0071 and 0.0125, respectively (two-tailed Student's t-test). (B) Concentration response of JH synthesis by AST-C. Two pairs of CA from day 2 4th instar larvae were cultured with varying concentrations of AST-C for 6 h, and then JH synthetic rate was measured. Each point represents Mean ± S.E. (N=4–5). [a,b]Significantly different at P=0.0066 and 0.0004, respectively (two-tailed Student's t-test). (C) Change in the mRNA expression profiles of AST-C receptor (BNGR-A1) in the CA during the 4th and 5th instar stadium. The expression levels were analyzed by the quantitative real-time PCR. Each point represents Mean ± S.D. (N=3).

that the suppressive action on JH biosynthesis by sNPF and AST-C targets the JH biosynthetic enzymes in the CA, and the mode of action of these peptides is different.

Allatotropin (AT) as a regulator for sNPF expression

AT is known to stimulate the JH synthesis of the *M. sexta* adult female CA (Kataoka et al., 1989), but *B. mori* AT showed no allatotropic action when assayed on JH synthesis in the CC–CA complex of day 3 4th and day 0 5th instar larvae (Fig. 5A). Yet, the expression of AT receptor and sNPF transcripts in the CC during the 4th and 5th larval stadia showed very similar patterns (Fig. 6A), so that some relationship between AT and sNPF might be present. When day 1 5th CA were cultured with 1 μM AT for 6 h, the transcript level of sNPF increased significantly (Fig. 6B), but AT had little effect on the sNPF expression in day 2 4th CC, indicating that AT is a stage-specific activator for sNPF expression.

Discussion

The control of JH synthesis is orchestrated by many inputs such as humoral factors from various organs/tissues and neuronal signals, and the actions of these factors are different in different developmental stages. Among important inputs the brain has a central role to regulate JH production in various insect species (Stay, 2000; Weaver and Audsley, 2009). As shown here, in *B. mori* the brain also controls JH synthesis by the CA (Fig. 1). It was apparent that the brain had a suppressive action which was due to the release of humoral factor(s) and/or neuronal connections, and one of the suppressive humoral factors was AST-C (Fig. 2). In addition, CC also played an important role on the JH synthesis by producing and releasing sNPF that also suppressed JH synthesis. Unlike AST-C, sNPF suppresses JH synthesis in a stage-specific fashion (Figs. 1 and 3).

Roles of AST and sNPF on the JH biosynthesis

ASTs are peptides produced by the neurosecretory cells in the brain that inhibit JH biosynthesis by the CA. Among three different types of ASTs (AST-A, -B, and-C), AST-C is found in the Lepidoptera (reviewed by Stay and Tobe, 2007; Audsley et al., 2008; Weaver and Audsley, 2009; Goodman and Cusson, 2012), Diptera and homologs in other insect orders (Veenstra, 2009). AST-C from the brain apparently suppressed JH biosynthesis in all the stages from the 4th to 5th instar stadium, and did not show any

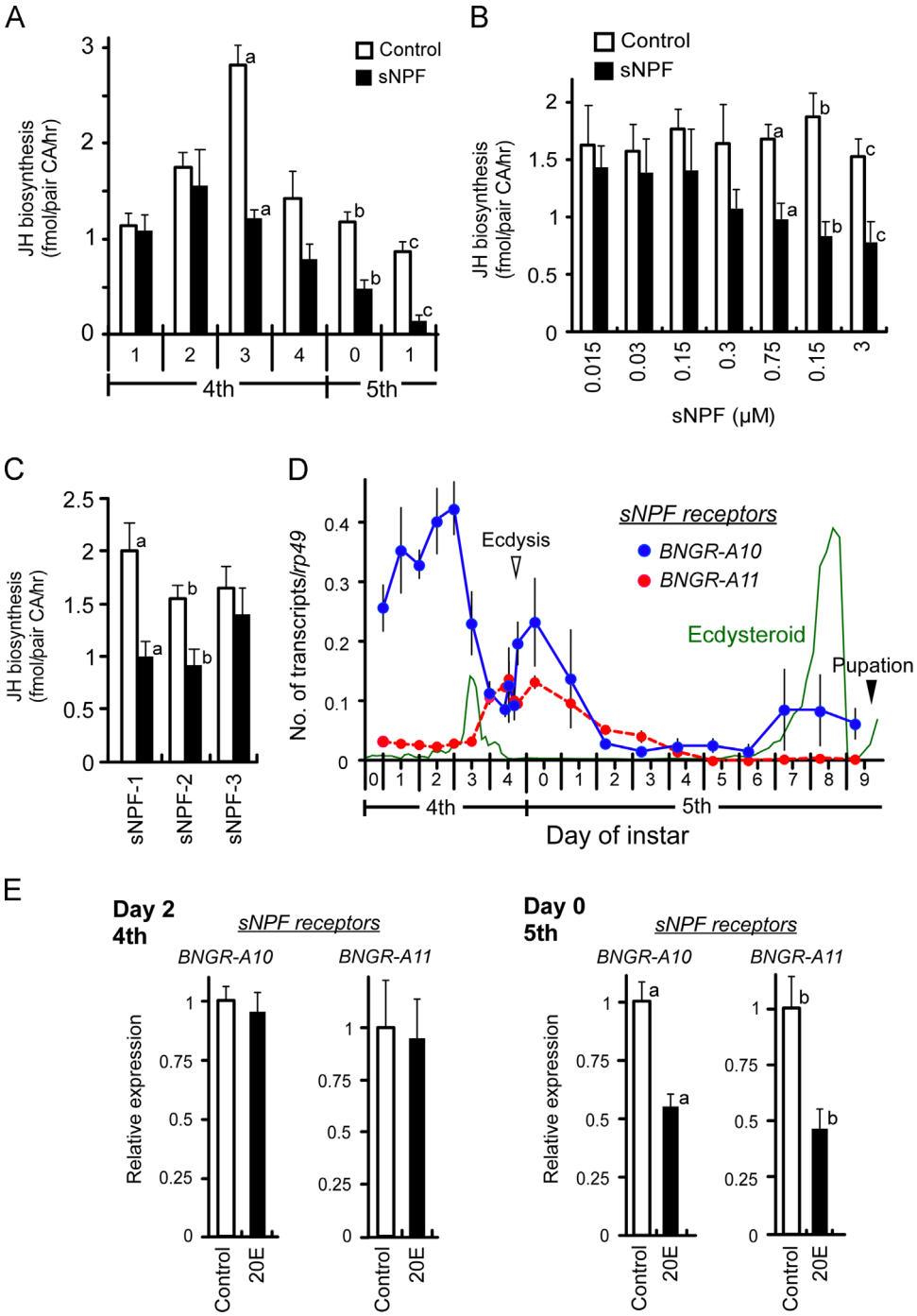

Fig. 3. Roles of sNPF on the JH biosynthesis and the regulation of its receptor expression in the CA. (A) Developmental changes in the inhibition of JH biosynthesis by sNPFs. Two pairs of CA dissected from each day from day 1 4th to day 1 5th were cultured with or without 3 μM sNPF for 6 h. Used sNPF was a mixture of 1 μM each sNPF-1, -2 and -3 [see (C); unless otherwise mentioned, mixtures containing the same amount of each isoform were used]. Each point shows Mean ± S.E. (N=3–6). a, b, and c are significantly different at P=0.0024, 0.0006 and 0.0002, respectively (two-tailed Student's t-test). (B) Concentration response of JH synthesis by sNPF. Two pairs of CA from day 0 5th instar larvae were cultured with varying concentrations of sNPFs for 6 h, and then JH synthetic rate was measured. Used sNPF was a mixture of each equal concentration of sNPF-1, -2 and -3 [see (C)]. Each point represents Mean ± S.E. (N=5–7). [a, b, c] Significantly different at P=0.0052, 0.0056 and 0.014, respectively (two-tailed Student's t-test). (C) Inhibitory activity of three isoforms of sNPF, sNPF-1, sNPF-2 and sNPF-3. Two pairs of CA from day 0 5th instar larvae were culture with 1 μM each sNPF. Each point represents Mean ± S.E. (N=5–6). a and b are significantly different at P=0.0165 and 0.0156, respectively (two-tailed Student's t-test). (D) Temporal changes of the expression of sNPF receptors (BNGR-A10 and BNGR-A11) in the CA during the 4th and 5th instar stadium. The expression levels were analyzed by the quantitative real-time PCR. Each point represents Mean ± S.D. (N=3). The ecdysteroid titer is from Muramatsu et al. (2008). (E) Effect of 20E on the expression of sNPF receptors. CA of day 2 4th or day 0 5th instar larvae were incubated with 0.5 μg/ml 20E for 6 h, and then the expressions of the receptors were determined by the quantitative real-time PCR. The expression levels without hormones were set as 1. Each point represents mean ± S.D. (N=5–7). a and b significantly different at P= < 0.0001 (two-tailed Student's t-test).

stage-specific variation in effect (Fig. 2A). The lower amount of JH synthesis by an intact brain–CC–CA complex (Fig. 1A) is due to AST-C as mentioned above, and the inhibitory action of AST-C is most likely through the axons from the brain rather than AST released in the hemolymph, as the effective concentration was relatively high in in vitro CA culture experiments (Fig. 2B) (> 0.5 μM vs. 0.01 μM; assayed with the Ca^{2+} imaging analysis using the HEK293 cells) (Yamanaka et al., 2008). No effect on the

JH synthesis by co-culture with isolated brains (Fig. 1B) supports this hypothesis. AST-A peptides that were first isolated from the cockroach *Diploptera punctata* and have been found in many different insect species, including Lepidoptera (Duve et al., 2003; Stay and Tobe, 2007). Cockroach AST-A peptides stimulate JH biosynthesis in a stage-specific fashion (Stay et al., 2002; Clark et al., 2008), but in *B. mori*, AST-C did not show any stimulative action (Fig. 2A). The role of AST-C on the JH biosynthesis of *B. mori* is probably to adjust the amount of JH synthesized to prevent its over-production, although the timing of the release of this peptide is unknown.

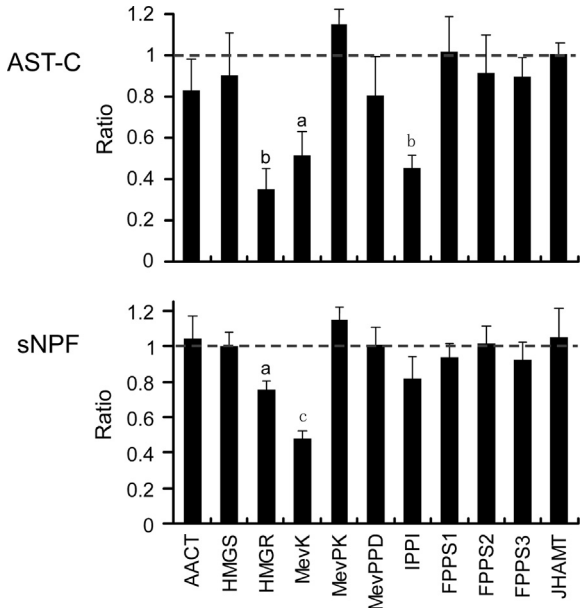

Fig. 4. Suppression of the JH biosynthetic enzymes by sNPF and AST-C. CA from day 0 5th were cultured with or without 1 μM AST-C or 3 μM sNPF (mixture of three isoforms) for 6 h, and then the expression levels of the JH biosynthetic enzymes were determined by the quantitative real-time PCR. The expression levels without hormones were set as 1. Each point represents mean ± S.E. (N=3–4).[a, b, c] are significantly different at P= < 0.05, < 0.01 and < 0.005, respectively (two-tailed Student's t-test).

The importance of CC in the regulation of JH biosynthesis had not been reported, but the CC apparently play an important role in regulating its synthesis by producing sNPF, and the suppressive action by the CC is likely due to sNPF that would normally be transported from the CC to the CA through the nerves. This is indicated by the fact that the effective concentration of sNPF

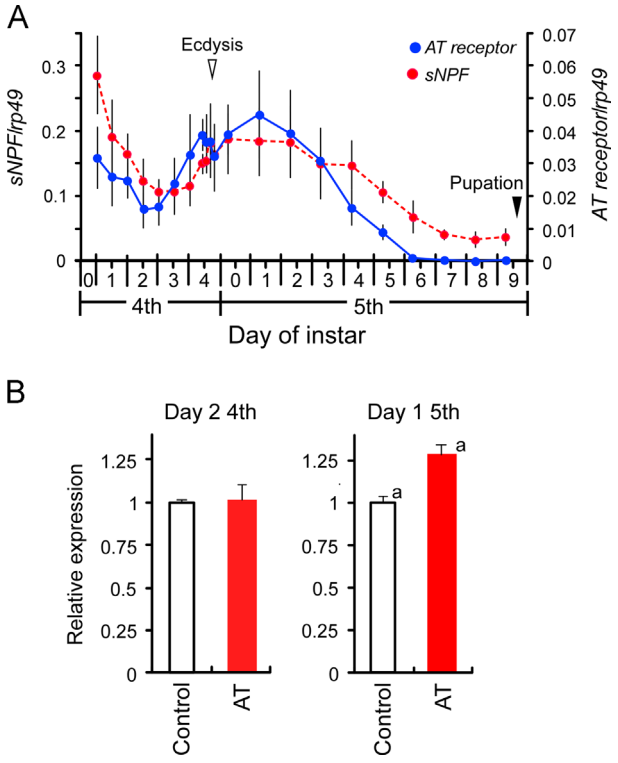

Fig. 6. Developmental transcript profiles of sNPF and the receptor for AT in the CC, and the effect of AT on sNPF expression. (A) Changes in the mRNA levels of sNPF and of the AT receptor in the CC during the 4th and 5th instar larvae. Each point represents Mean ± S.D. (N=3). (B) Stimulation of sNPF expression by AT. The expression level without AT was set as 1. CC of day 2 4th or day 1 5th were incubated with or without 1 μM AT for 6 h, and then the amounts of sNPF mRNA were analyzed by the quantitative real-time PCR. Each point represents mean ± S.D. (N=3–4). [a] Significantly different at P=0.0143 (two-tailed Student's t-test).

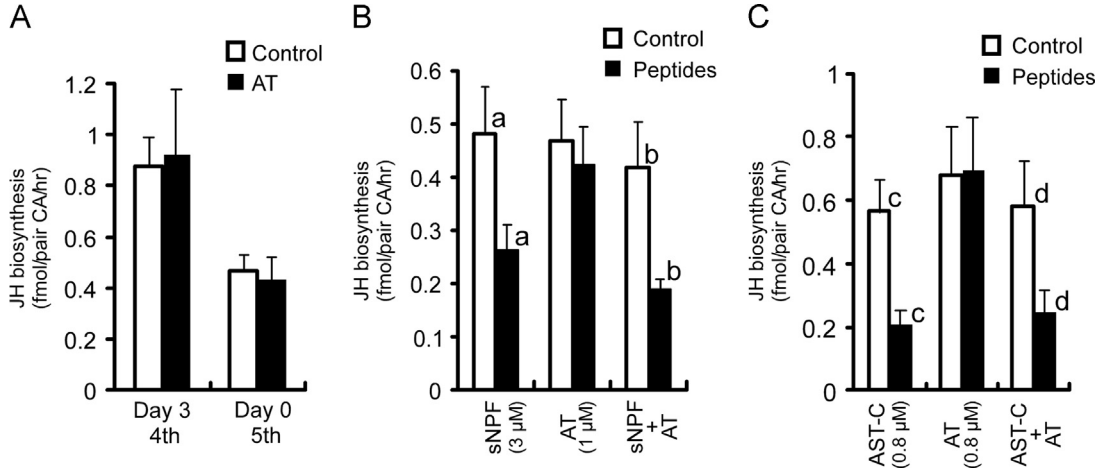

Fig. 5. Effect of AT on the suppressive action of JH biosynthesis by sNPF (3 μM) and AST-C (1 μM). (A) AT had little effect on the JH biosynthetic rate. Two pairs of CC–CA dissected from day 3 4th and day 0 5th were cultured with or without 1 μM AT for 6 h, and then JH biosynthetic activity was measured by RCA. Suppressed JH biosynthetic rate by sNPF (B) and AST-C (C) was not rescued by AT. Day 0 5th CC–CA complexes were cultured with or without peptides for 6 h, then JH biosynthetic rate was determined. 3 μM sNPF is a mixture of three isoforms (1 μM each). Each point represents mean ± S.E. (N=4–5).[a, b, c, d] Significantly different at P=0.0366, 0.0326, 0.0187 and 0.0404, respectively (two-tailed Student's t-test).

affecting on cells was high; $>0.75\,\mu M$ (Fig. 3B) compared with >1 nM that was effective with Ca^{2+} imaging using the HEK293 cells expressing the sNPF receptor, BNGR-A10 (Yamanaka et al., 2008). The lack of effect on the JH synthesis by co-culturing CC (unpublished) and the immunocytochemistry results of sNPF in the *B. mori* cephalic organs (Yamanaka et al., 2008) support this hypothesis.

Although the levels of sNPF in the hemolymph are unknown, the transcript levels of sNPF in the CC are relatively high with some fluctuations during the 4th instar and in the feeding stage of the 5th instar stadium (Fig. 6A). There are two putative isoforms of sNPF receptors, isoform 1 (BNGR-A10) and isoform 2 (BNGR-A11) (Yamanaka et al., 2008), and both are expressed in the CA. The expressions of both receptors became high in day 0 5th larval stadium at the time of which corresponded to the suppressive action of sNPF (Fig. 3A, D); therefore one of the causes to drive the stage-specificity by sNPF might be due to the up-regulation of the receptor expression, in particular, after the last larval ecdysis. Innervation of the CA to the brain eliminated this stage-specific variation by decreasing the overall JH synthesis.

20E suppressed the expression of the putative sNPF receptors, BNGR-A10 and BNGR-A11, in the CA of the day 0 5th instar larvae, but not in the CA from the day 2 4th. The suppression of the receptor expression by 20E in day 0 5th indicates that the decline of the ecdysteroid titer at the time of ecdysis (Kaneko et al., 2011b) allows the increase of their expression of sNPF to ensure that sNPF suppresses the JH synthesis after the last larval ecdysis. Unlike in day 0 5th larval stadium, the depression of the receptor expression in day 2 4th larval stadium is not regulated by 20E, so that other factors are involved in its regulation, but the mechanisms are yet to be solved.

Different modes of action of sNPF and AST-C suppressing JH biosynthesis

AST has been believed to inhibit JH synthesis by the transfer of citrate to the cytoplasm across the mitochondrial membrane or by the cleavage of citrate to yield cytoplasmic acetyl-CoA rather than by the inhibition of the JH synthetic enzymes (Sutherland and Feyereisen, 1996), but it is apparent that the inhibition of some of the JH synthetic enzymes is also involved. Both sNPF and AST-C suppress JH biosynthesis, but their mode of action was slightly different; expression of MevK and HMGR mRNAs expressions were suppressed by sNPF, although expression of these two enzymes, plus IPPI expressions was depressed by AST-C (Fig. 4). It is yet to be studied whether or not the two neuropeptides share the same cell signaling pathway downstream of their receptors. The expression levels of most of the mevalonate enzymes and of JHAMT showed a decline in day 3 4th instar stadium and after the last larval ecdysis (Kinjoh et al., 2007); therefore the stage-specific suppression of JH synthesis is not only due to the inhibition of the HMGR and MevK expression by sNPF but also additional factors such as the stage-specific suppression of the expression of its receptor (Fig. 3D), together with other mechanisms such as the transportation of citrate to yield acetyl CoA (Sutherland and Feyereisen, 1996) are likely involved.

AT is a stage-specific regulator for sNPF in a larval stadium

AT activates only the CA of female adult *M. sexta*, and the mosquito *Aedes aegypti* (Kataoka et al., 1989; Li et al., 2003), although AT is expressed in larval brains (Duve et al., 2003; Nagata et al., 2012). It is also interesting to note that in a sphingid moth *Agrius convolvuli*, AT activated JH synthesis by CA from adult female in vitro as found in *M. sexta*, but this was only seen at high incubation temperatures such as 30 °C, which was the temperature that the original paper (Kataoka et al., 1989) reported, but AT did not activate its synthesis at 25 °C (Hiruma and Kaneko, 2013). In addition, AT did not activate CA of other stages or males of *A. convolvuli* even at 30 °C.

In *B. mori*, AT had little effect on the JH biosynthesis in the larval CC–CA complexes, and the addition of the inhibitory peptides to AT, such as sNPF and AST-C, did not affect their inhibitory action (Fig. 5), so that AT did not seem to have any functions on the JH synthesis. Yet the expression of the AT receptor occurs in the same cells in the sNPF producing cells in the CC, and not in the CA (Yamanaka et al., 2008). In addition, the expressions of the AT receptor and sNPF in the CC are mirrored in both 4th and 5th instar stadium (Fig. 6A). AT was found to increase sNPF expression only in day 1 5th instar larvae, a time that sNPF was needed to inhibit JH biosynthesis and not in day 2 4th (Fig. 3, Fig. 6B). Therefore, AT is a stage-specific stimulator for sNPF and this specificity appears to be involved in the stage-specific suppression of JH synthesis by sNPF. Since AT had little effect on the JH synthesis by larval CC–CA complexes with or without sNPF in vitro (Fig. 5A, B), AT does not have a function to cause transportation of sNPF to CA from CC, so that JH synthesis is little affected.

sNPF is one of the important factors to initiate pupal metamorphosis

In the last larval stadium, larvae prepare for pupal metamorphosis, which is characterized by the larval–pupal commitment of various tissues such as epidermal cells (Riddiford, 1985, 1996; Muramatsu et al., 2008). This event is the first step for pupal metamorphosis and the removal of the JH in the hemolymph is indispensable for this process. Indeed the cessation of the JH synthesis by the CA is one of the most important contributions. Four factors have been identified to suppress the JH biosynthesis in the early 5th (last) instar stadium in *B. mori* that include dopamine (Hiruma and Kaneko, 2013), sNPF (Fig. 3), AST-C (Fig. 2) and the decline of ecdysone titer (Kaneko et al., 2011b). To guarantee to shut-down the JH synthesis, these factors have different modes of action; dopamine acts directly through a dopamine receptor in the CA, sNPF and AST-C suppress the expression of some of the JH biosynthetic enzymes (Fig. 4), and 20E acts via the brain and this signal transfers to the CA through the nerves to regulate JHAMT, IPPI and FPPS1 expression. In addition to the cessation of the JH biosynthesis by CA, the degradation of the JH in the hemolymph is facilitated by various types of JH esterases (Tsubota and Shiotsuki, 2010), so that pupal metamorphosis is initiated.

Although JH and its precursors are key hormones for arthropod molting and metamorphosis, the studies on the precise regulatory mechanisms of its biosynthesis had been hampered by the minute structure of the CA and the sticky nature of JH. By challenging this issue, part of the network of peptide hormones engaging JH biosynthesis has been uncovered in this study and more detailed analyses will be necessary to construct the comprehensive network of its synthetic machinery including the interactions with other factors.

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